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13. ABSTRACT (Maximum 200 Words)

Fluid and electrolyte balance is affected differently at different stages of alcohol use. In this study, we examine the role of vasopressin (VP), an important hormone in body fluid regulation, in the physiological response to alcohol. A transient decrease in circulating VP levels occurs immediately after acute alcohol administration, but VP levels return to baseline levels while alcohol is still present in the blood, and there is no prolonged deficiency of VP associated with blood alcohol levels. Rather, alcohol-induced changes in renal responsiveness to VP appear to be responsible for the pattern of diuresis, impaired water excretion, and recovery in the different phases of alcohol exposure. Up- and down-regulation of renal VP V2 receptors involved with renal tubular water reabsorption cause differentially altered renal function in the different phases of alcohol exposure. Further, this alcohol-induced renal receptor regulation is specific to the V2 receptors in the inner medulla region of the kidney. In addition, chronic alcohol exposure disrupts the relationships between VP synthesis, brain VP V1 receptors, and blood tonicity. Thus, alcohol-induced changes in VP regulation may affect the ability to respond to physiologic stimuli. Sensitivity of the VP system is currently being further examined with salt load stimulation studies.

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INTRODUCTION:

Alcohol use impairs renal fluid handling and the ability to maintain adequate hydration. Of importance from a military readiness aspect, is that alcohol exposure causes physiological changes in fluid and electrolyte balance that will affect soldier performance. The soldier who uses alcohol is even more susceptible to dehydration especially when water is scarce, and from a pharmacological perspective, would be more susceptible to exposure to chemical warfare agents that would reach toxic levels in the dehydrated alcohol user faster than an individual with adequate hydration.

Fluid and electrolyte balance appears to be affected differently at different stages of alcohol use. In this study, the role of vasopressin, an important hormone in body fluid regulation, in the physiological response to alcohol is being examined. In rat models of acute and chronic alcohol exposure, we are taking a systematic approach at elucidating the relationship between vasopressin synthesis in the brain, receptor regulation in the kidneys, and water and salt handling during different phases of alcohol exposure.

Our results to date have provided evidence of water imbalance with alcohol exposure that is due to altered numbers of vasopressin receptors, specifically renal V2 receptors, involved with tubular water reabsorption. Additionally, the relationships between vasopressin synthesis, brain vasopressin receptors and blood tonicity appear to be disrupted by chronic alcohol exposure.

The results of this research will lead to better strategies for management of fluid and electrolyte imbalance associated with alcohol use and will benefit military operational readiness by helping to provide medical countermeasures for soldiers who use alcohol.

PROGRESS IN YEAR 3:

We have accomplished the original goals of 1) evaluating fluid and electrolyte regulating ability in models of acute and chronic alcohol exposure and alcohol withdrawal, and 2) searching for mechanisms of altered fluid handling. During this three year project we have uncovered mechanisms behind altered fluid handling in different stages of alcohol exposure. In the process, we have developed well-characterized models of acute, chronic, and withdrawal from alcohol exposure that all exhibit similar alterations in fluid handling as found in studies of alcohol in humans. These models can be used to define mechanisms behind alcohol effects better than study of humans, however, because conditions of alcohol dosing, hydration status, and fluid intake and output can be better controlled and monitored. We have used sensitive real-time polymerase chain reaction (qPCR) assays that we have developed for quantitation of mRNA for vasopressin and vasopressin receptor syntheses to reveal the relationship between physiologic stimuli of vasopressin and vasopressin receptor synthesis that could not before be seen via less sensitive traditional methods that could not detect fine changes in peptide levels.

In this third year, we have verified preliminary findings previously reported in the last 2 annual reports, and re-evaluated directions of the research originally proposed based on those findings. We have also identified new lines of research that need to be further explored with regards to the effect of alcohol exposure on vasopressin responsiveness to physiological stimuli.

Research accomplishments associated with each task outlined in the Statement of Work are as follows:

1. *Fluid and electrolyte regulating ability experiments*

Animal models: We continue to use and characterize our animal models of acute and chronic alcohol exposure and withdrawal from alcohol, and these models continue to yield consistent, dependable responses. We continue to take advantage of the ability to run several experiments in the same animal that allows considerable reduction of the total numbers of animals used. This allows for control of variability between individual animals, and enables the generation of data with greater precision and detection of finer differences in physiologic regulation of fluid balance between groups. This repeated measures design also provides for closer comparison of several arms of the experiments and simultaneous assessment of multiple aspects of fluid regulation.

The alcohol models studied thus far have been acute alcohol exposure, chronic alcohol exposure and 4 weeks of withdrawal. Because the alcohol dose chosen for these experiments was moderate (equivalent to about 2 six packs of beer a day in an adult human) the alcohol affects observed were not permanent. Our withdrawal group thus showed signs of recovery from altered fluid balance by 4 weeks of removal from alcohol and it was not necessary to study a "late phase" of alcohol withdrawal (SOW model "d"). In addition, as reversal of chronic alcohol effects occurred by simple removal of alcohol exposure, it was not necessary to examine models of "treatment" with V2 agonist or antagonist during the withdrawal phase. Also, findings from our V2 agonist and antagonist dose response experiments, as well as findings that there were no differences in circulating vasopressin levels in our models of chronic alcohol and alcohol withdrawal, made it no longer reasonable to treat with V2 analogues to normalize circulating vasopressin, as originally proposed in SOW models "e" and "f". (It would still be interesting to develop more severe models of chronic alcohol exposure that would cause long-lasting effects or

permanent tissue damage that was not evident in our models. We hope to pursue this different aspect of alcohol tissue injury perhaps in another future grant project.)

Therefore, instead of studying pharmacological treatment during withdrawal as we originally proposed in SOW rat models "e" and "f", in this third year of this project, as it was no longer logical to do so, we have instead replaced that series with more thorough examination of the responses to the immediate pharmacological effect of alcohol during alcohol diuresis. We thus compared the immediate pharmacological effect of alcohol with our previous results describing the after-effects of acute alcohol once alcohol blood levels were no longer evident. Despite the traditionally accepted concept that alcohol inhibits vasopressin release and that it is the inhibition of circulating vasopressin levels that causes alcohol-induced diuresis, there are numerous reports that vasopressin levels are unchanged or even elevated after alcohol ingestion. Although circulating vasopressin levels have been measured in humans and animal models of alcohol exposure in several studies, only a few studies have ever actually documented a decrease in vasopressin circulating levels (Helderman et al, 1978; Eisenhofer and Johnson, 1982; Le-papaluoto et al, 1992), and even these have only shown just a very short term suppression at best.

While the majority of other studies have not been able to demonstrate a decrease in vasopressin levels, this has been attributed to the difficulty in detecting a suppression of already low basal vasopressin values with most vasopressin assays. Further, state of hydration of study subjects, nausea, stress due to the method and dosage of alcohol administration, or the use of anesthetized animal models, could all account for frequent reports of increased vasopressin levels after alcohol. Still, there was the possibility that perhaps vasopressin does indeed increase after alcohol as many of these reports indicate, and if so, vasopressin could not be entirely responsible for alcohol-induced diuresis.

We therefore felt it necessary to determine what was truly occurring with vasopressin levels and urine flow during elevated blood alcohol levels, especially since much of the acceptance that suppressed vasopressin levels are responsible for alcohol-induced diuresis of dilute urine is based on inference that vasopressin is the main hormone involved in renal water regulation, and indirect evidence that alcohol-induced diuresis can be prevented or reversed with administration of exogenous vasopressin. This conclusion from indirect evidence could be faulty, however, as vasopressin administration could also decrease urine flow in diuresis not necessarily caused by vasopressin suppression. We thus further characterized our animal model of acute alcohol exposure, by examining the time course profiles of blood alcohol levels (fig. 1), vasopressin levels (fig. 2), and diuresis (fig. 3) during alcohol exposure.

Blood alcohol levels immediately increased after bolus intragastric (i.g.) administration of alcohol, and vasopressin levels did decrease and were significantly lower than baseline 30 minutes after alcohol, but returned to baseline levels by 60 minutes. This was in agreement with studies that showed an immediate but transient decrease in vasopressin levels in humans (Helderman et al, 1978). This is in contrast to other studies utilizing anesthetized animal models (Cooper and Musabayane, 2000) where we suspect alcohol caused a decrease in blood pressure and renal hemodynamics resulting in increased vasopressin levels and antidiuresis. Thus our conscious animal model which enables better study of renal function without the confounding effects of anesthesia or surgical stress, we believe, reflects the true effect of alcohol on vasopressin release and renal action. Also, examining the time course of changes in blood alcohol, circulating vasopressin, and urine flow after a single i.g. bolus administration of alcohol allowed observation of the relationships between these changes better than a continuous

intravenous infusion of alcohol that can also cause changes in blood pressure and hemodynamics.

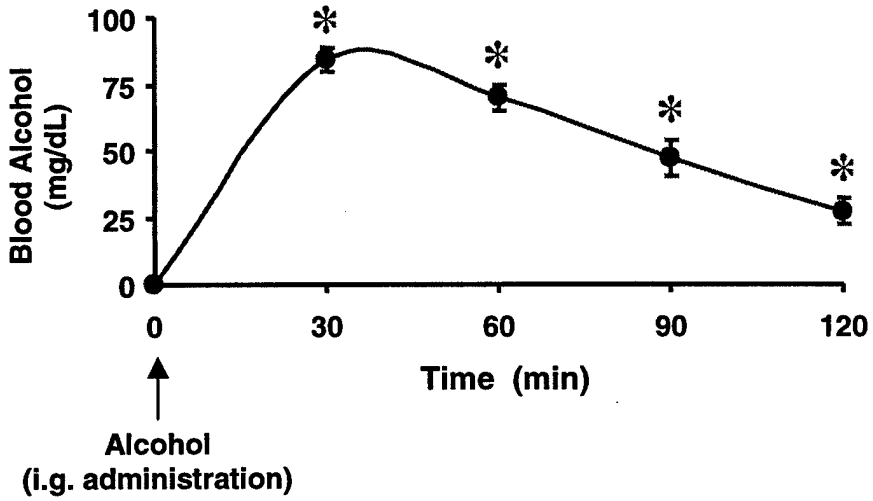

fig. 1 Time course of blood alcohol levels. Blood alcohol levels peak as early as 30 minutes after a single intragastric bolus of ethanol (15% v/v ethanol in water 1ml/100g body weight) and are still elevated 120 minutes after alcohol dosing. (Values represent mean ± s.e.m. n=22. * = different from time zero, $p<0.05$.)

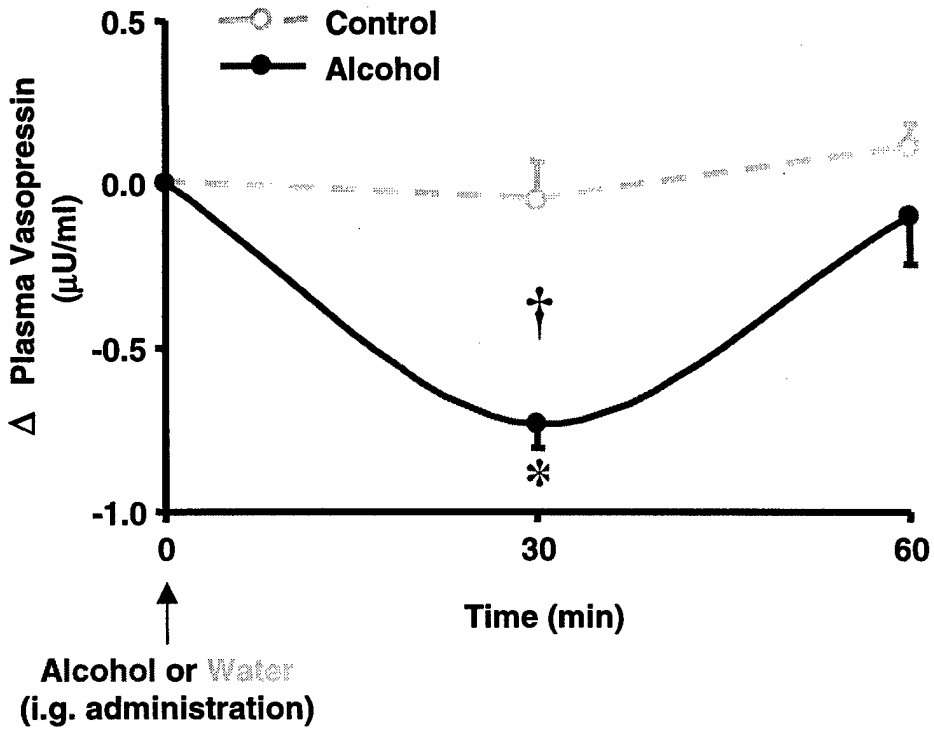

fig. 2 Time course of circulating vasopressin levels. A single intragastric bolus of ethanol (15% v/v ethanol in water 1ml/100g body weight; n=8) caused a transient decrease in plasma vasopressin levels at 30 minutes compared to water administration (n=7), but returned to baseline by 60 minutes. (Values represent mean ± s.e.m. * = different from time zero, $p<0.05$. + = different from control, $p<0.05$)

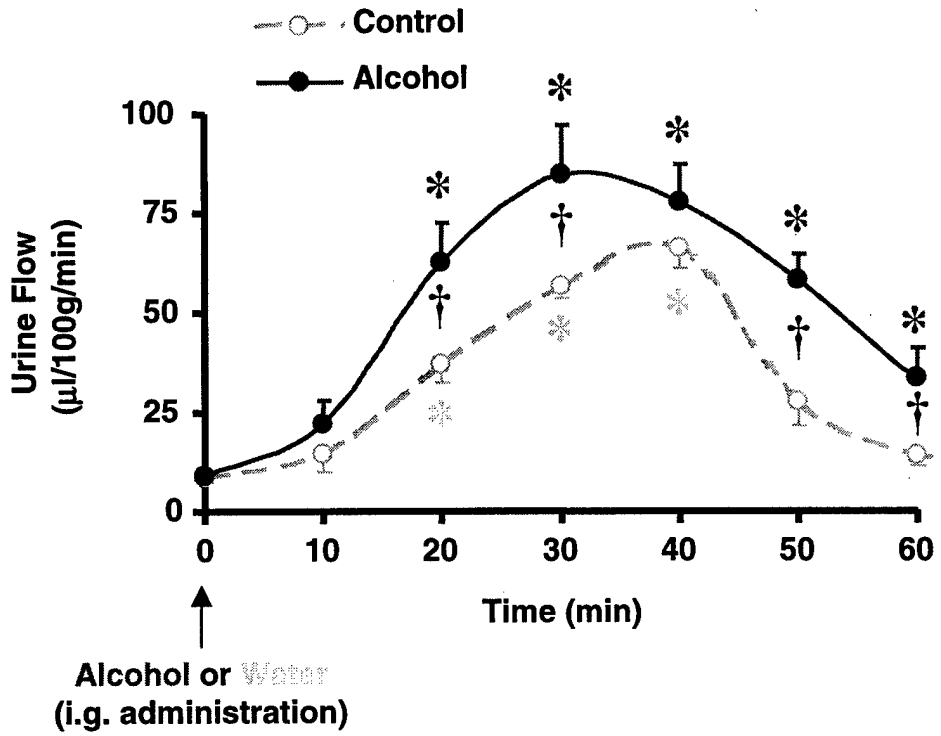

fig. 3 Time course of diuresis. A single intragastric bolus of ethanol (n=6) caused an increased diuresis compared to water administration in control group (n=6). Time course of increased urine flow was similar to that seen with vasopressin levels with urine flow peaking at 30 minutes and returning toward baseline by 60 minutes. (Values represent mean ± s.e.m. * = different from time zero, $p<0.05$. + = different from control, $p<0.05$)

Thus, the time course of diuresis indicates that while acute alcohol exposure may disrupt VP release momentarily, there is no prolonged deficiency of VP associated with blood alcohol levels. Rather, a brief decrease in circulating VP after alcohol intake causes an immediate diuresis. Prolonged diuresis associated with renal V2 receptor down regulation as we have previously shown, is likely responsible for elevated VP levels reported as an after effect of alcohol.

Examination of the ability of the kidneys to excrete a water load:

Experiments testing the ability to excrete a water load for all three models of acute alcohol exposure, chronic alcohol exposure, and withdrawal from alcohol have been completed. We have confirmed our previous reports of 1) acute alcohol exposure increasing water diuresis over 18 hours after the last alcohol intake, even after blood alcohol levels are undetectable; 2) an impaired ability in rats with chronic alcohol exposure to excrete a water load; and 3) a reversal of the impaired water load excretion ability 4 weeks after removal of alcohol. In all three phases of alcohol exposure, a difference in vasopressin secretion does not appear to be responsible for effects on water excretion, as circulating vasopressin levels were not different in any of these phases. We have now confirmed our previous preliminary findings that the mechanism behind

altered renal water handling ability is a difference in renal responsiveness to vasopressin. Accordingly, we have solidified the conclusions we proposed at the end of year 2. Specifically, we have determined that regulation of renal vasopressin V2 receptor gene expression (as demonstrated by changes in vasopressin V2 receptor mRNA in the inner medulla, and renal V2 receptor binding) rather than altered circulating levels of vasopressin is responsible for the differential water load excretion abilities at different phases of alcohol exposure.

V2 antagonist dose response curve generation to examine the renal response to endogenous vasopressin:

We postulated that alcohol might alter renal handling of fluid in acute and chronic alcohol use by affecting regulation of renal V2 receptors involved with tubular water reabsorption. To examine the whole animal effects of putative V2 receptor up or down regulation, as reported in progress report for year 2, we have completed V2 receptor antagonist dose response experiments in acute alcohol exposure, chronic alcohol exposure, and alcohol withdrawal models. With acute alcohol exposure, an alteration in V2 antagonist effect could not be demonstrated. This was likely because with acute alcohol exposure the kidneys may have not yet adapted with a long-lasting change in renal sensitivity to acute changes in endogenous vasopressin levels.

Final results in year 3 confirm the early results reported last year where, in accordance with their impaired ability to excrete a water load, rats chronically exposed to alcohol showed a blunted diuresis and a rightward shift of the dose-response curve to V2 antagonist inhibition of endogenous vasopressin. The suppression of V2 antagonist efficacy in increasing urine flow was due to attenuation of free water clearance in the chronic alcohol group. This decrease in V2 antagonist efficacy occurred despite no apparent differences in plasma vasopressin levels in these rats. Such results are consistent with the hypothesis that impaired ability to excrete a water load and a SIADH-like phenomenon of water retention in chronic alcohol users are due to altered renal responsiveness to endogenous vasopressin. As we have shown, an up regulation of vasopressin receptors in response to long-term alcohol exposure occurs, similar to that seen with long-term exposure to vasopressin antagonists (Caltabiano and Kinter, 1991) to compensate for the initial acute alcohol-induced diuresis effect. A greater number of receptors available to bind endogenous vasopressin, require greater amount of antagonist to compete for binding sites, and thus shifts the dose-response curve.

V2 antagonist dose response experiments with the withdrawal model were also completed in year 3. In contrast to results of the chronic alcohol exposure model, during withdrawal from alcohol, the response to a V2 antagonist returns to control responses. This is consistent with our findings that renal V2 receptor mRNA expression returns to control values during withdrawal. Thus, a putative retention of water during withdrawal does not seem to be the result of a persistent change in renal responsiveness to vasopressin.

V2 agonist dose response curve generation to assess maximum urine concentrating ability with maximal stimulation of vasopressin V2 receptors:

Because altered water handling in alcohol exposed rats may be due to an alteration of the renal medullary interstitium tonicity in these animals, the urine concentrating abilities in the face of maximal vasopressin V2 receptor stimulation in these rats were examined. In year 3 we have completed dDAVP dose-response experiments and have verified that there is no difference in maximal urine concentrating ability between the rats chronically exposed to alcohol and control

rats. Thus, these results show that there is no difference in the concentration gradient for fluid reabsorption and that altered fluid handling observed after chronic alcohol exposure is primarily due to differences in V2 receptor density. In year 3 we have also confirmed that the maximum urine concentration ability in response to maximal V2 stimulation with dDAVP is lower in rats experiencing alcohol withdrawal than in control rats. This suggests that during this phase of withdrawal, renal medullary tonicity is altered. The mechanism of this effect needs to be further investigated in future studies.

Examination of the stimulation of vasopressin release in response to a salt load:

We have run experiments and are currently analyzing samples to examine whether vasopressin response to physiologic stimuli such as a salt load may be altered with alcohol exposure. Preliminary results so far suggest that the relationship between baseline circulating vasopressin levels and plasma osmolality during withdrawal from alcohol is altered. We compared the secretion of VP in response to an osmotic stimulus between rats after alcohol withdrawal (n=5) and rats never exposed to alcohol (control, n=5). After basal circulating VP (pVP) and plasma osmolality (pOsm) values were obtained from conscious resting rats, blood was sampled at 20, 40, and 60 minutes during a hypertonic saline ramp (5% NaCl i.v. at 10 µl/100g/min). Samples were used to generate pOsm-pVP curves. The curve generated in the withdrawal group was shifted to the left of controls (fig. 4). This increased stimulation of VP secretion during alcohol withdrawal is consistent with the idea of rebound VP secretion creating a condition similar to a syndrome of inappropriate antidiuretic hormone secretion (SIADH) that has been proposed to explain water retention problems reported in chronic alcoholics (Trabert *et al*, 1992).

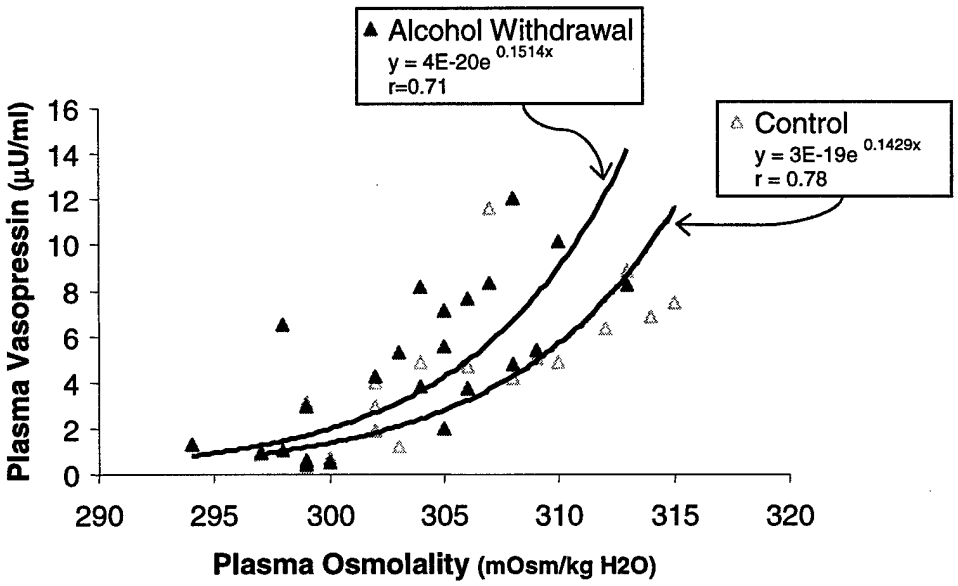

fig. 4 Relationship between plasma osmolality and plasma vasopressin levels in rats during alcohol withdrawal phase. The pOsm-pVP curve of withdrawal animals is shifted to the left of control animals, indicating a hypersensitivity of vasopressin release during withdrawal. (Curves generated from data from 5 rats in each group.)

Assessment of vasopressin clearance to assess the influence of alcohol on vasopressin metabolism:

We have demonstrated that the altered fluid handling with alcohol exposure can be attributed to altered renal sensitivity to vasopressin, as evidenced by renal receptor mRNA and binding data. However, we continue to explore the relationship between vasopressin synthesis and release by the brain into the circulation and will continue examining the factors regulating this steady vasopressin circulating level by examining vasopressin clearance. It is possible that vasopressin metabolism by the kidney may be altered, and even if there is a change in brain vasopressin mRNA expression and vasopressin synthesis, circulating levels remain unchanged because vasopressin clearance also changes accordingly. If renal vasopressin receptors are affected by chronic alcohol exposure, it is likely that clearance of vasopressin from the circulation is also affected. This is in accordance with the theory that vasopressin renal clearance is receptor mediated (Keeler et al., 1991).

Vasopressin clearance assessments in acute, chronic, and withdrawal models are ongoing. We have collected samples from chronic and withdrawal models, and are currently developing better methods of sample handling and extraction of vasopressin from urine so that our assay results are not confounded by interfering measurements of urinary metabolites. This fine-tuning of our radioimmunoassay for urinary vasopressin is essential in order to obtain accurate clearance values.

2. *In vitro assessments of tissues and samples to elucidate mechanisms behind altered fluid handling*

Measurement of vasopressin levels in the pituitary, blood, and urine:

Chronic alchoholism associated with water retention is supposedly due to increased circulating vasopressin or no change in vasopressin levels but an increase in renal vasopressin sensitivity, impaired renal water excretion, hyponatremia, and cirrhosis of the liver. Alcohol withdrawal, especially in patients with delirium tremens (Trabert et al., 1992) is linked to an increased plasma vasopressin concentration believed to be the result of rebound secretion of vasopressin.

Mean values of baseline plasma osmolality (pOsm), plasma vasopressin (pVP), pituitary vasopressin (pit VP), VP mRNA, V1R mRNA, and V2R mRNA of control and chronic alcohol-exposed animals were compared (fig. 5). There were no statistically significant differences detected in basal levels. We felt, however that it was probably more the relationships between these variables that needed to be examined rather than a snapshot of baseline levels.

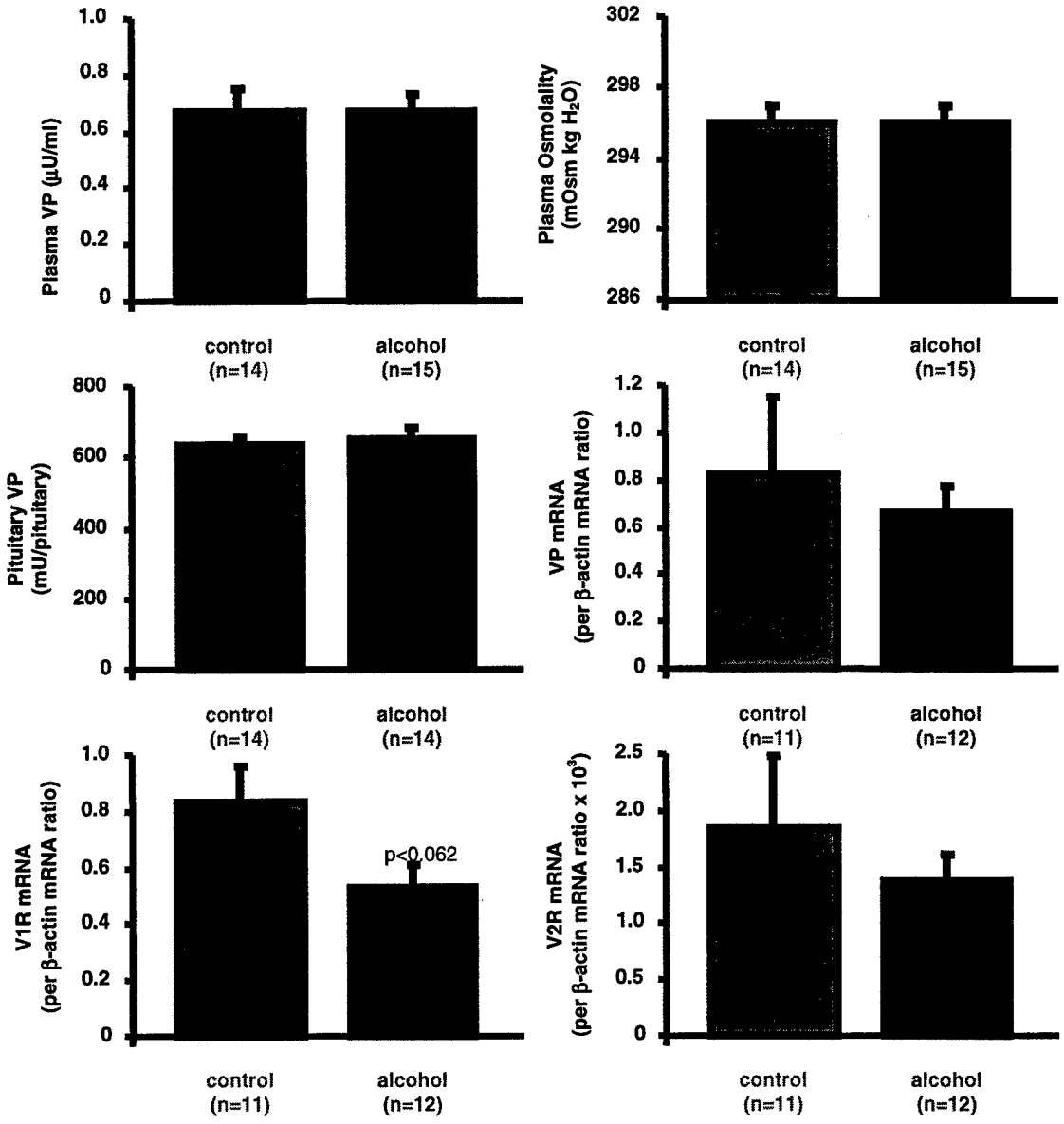

fig. 5 Comparison of resting baseline plasma and pituitary vasopressin levels and brain mRNA gene expression. There were no differences in plasma osmolality, plasma vasopressin, pituitary vasopressin content, brain VP mRNA, or brain V2R mRNA baseline values between control and alcohol groups. Brain V1R mRNA was not significantly different at the $p<0.05$ level, but there appeared to be a depression of the mRNA levels in most of the alcohol-exposed animals compared to controls. Values represent means $\pm$ s.e.m.

Measurement of brain vasopressin and vasopressin receptor mRNA

The relationship between vasopressin gene expression in the brain, synthesis, and release has not been systematically studied during the various phases of alcohol exposure and withdrawal. Thus, in this third year we further characterized the relationship between vasopressin synthesis, brain V1 receptor regulation, and osmolality.

The relationships between vasopressin synthesis (VP mRNA), vasopressin release (pituitary VP, plasma VP, plasma osmolality), and vasopressin receptor regulation (V1 mRNA, V2 mRNA) in the brain were examined utilizing stepwise multiple regression to identify independent predictors for each of the variables. Predictor variables that showed a clear contribution to the regression equation independent of other variables were then further examined with simple linear regression, and curve fitting was done to achieve the best correlation coefficient. Stepwise multiple regression showed that the only independent predictor of VP mRNA was brain V1R mRNA. Thus, somehow, vasopressin synthesis was associated with V1 receptor up regulation. Both brain VP mRNA (fig. 6) and plasma osmolality (fig. 7) each significantly contributed independently to a relationship with V1RmRNA in control rats but not chronic alcohol-exposed rats.

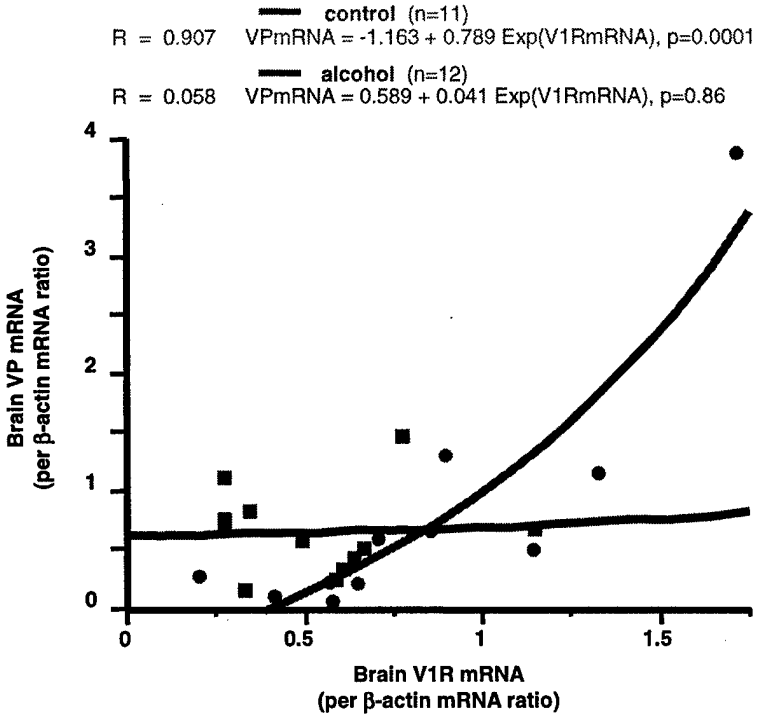

fig. 6 Relationship between brain V1R mRNA and VP mRNA. Relationship curve generated from baseline steady state values and evident in the normal physiological variability between rats. The relationship between brain V1 mRNA and VP mRNA was only evident in control rats, and did not exist in alcohol-exposed rats.

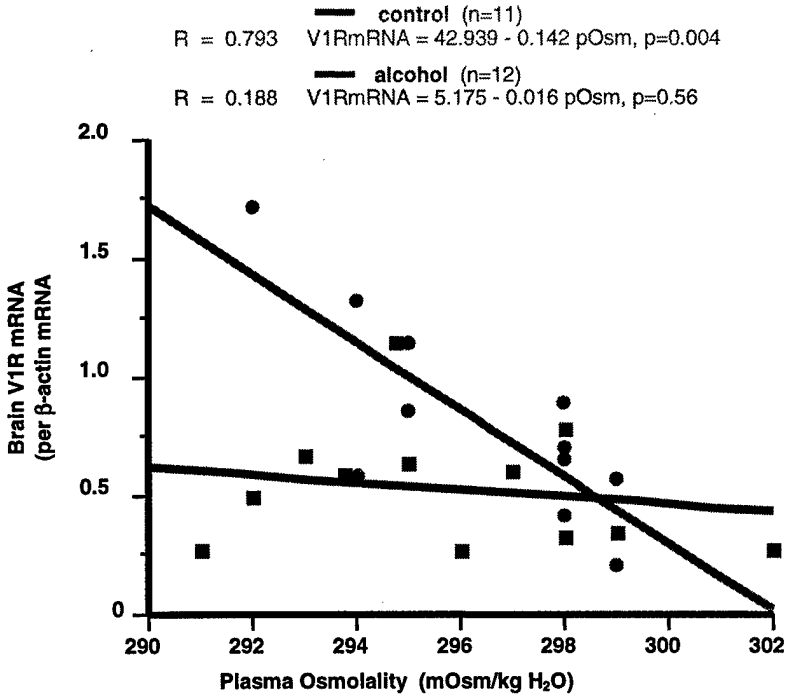

fig. 7 Relationship between plasma osmolality and brain V1R mRNA. Relationship curve generated from baseline steady state values and evident in the normal physiological variability between rats. The relationship between plasma osmolality and brain V1 mRNA was only evident in control rats, and did not exist in alcohol-exposed rats.

The strong correlations between V1RmRNA, VP mRNA, and plasma osmolality, do not reveal the causal relationships between these variables. These results do indicate that brain V1R generation is highly sensitive to slight changes in plasma osmolality, and that the V1 receptor may serve as an osmotic sensor involved in the stimulation of vasopressin synthesis. Stepwise multiple regression screening of independent predictors seems to suggest a scenario where V1 receptors may participate in a positive feedback mechanism which helps amplify a vasopressin response. Thus, some stimulus causes VP mRNA to be up regulated, which induces vasopressin synthesis and an increase in circulating vasopressin. This results in action on the kidney and a resultant decrease in plasma osmolality, which causes up regulation of V1 receptors, which in turn continues to stimulate VP mRNA and vasopressin synthesis.

Regardless of the actual roles V1 or V2 receptors play in the mediation of vasopressin synthesis, it is clear from this study that alcohol disrupts all normal relationships between osmotic signals, vasopressin synthesis and release, and vasopressin receptor regulation. An uncoupling of vasopressin secretion and release into the circulation has been indicated in at least one previous study where plasma vasopressin levels and plasma osmolality were shown to be increased after alcohol exposure while hypothalamic vasopressin mRNA remained unchanged (Hoffman and Dave, 1991). An inability of vasopressin synthesis to appropriately respond to physiological signals likely contributes to the body fluid imbalances seen in alcoholism. If the role of specific vasopressin receptor subtypes in the mediation of vasopressin synthesis, as suggested by our data, can be further elucidated, pharmacological strategies may be developed to correct fluid regulation problems associated with this chronic disease.

Additional assessment of the relationship between vasopressin brain content and blood levels is ongoing to help us define causal relationships in this apparent uncoupling of vasopressin synthesis and physiologic stimuli that occurs with alcohol exposure. We have also established in our lab over the last year, the ability to study an isolated explant of a hypothalamus preparation with the pituitary stalk and neurohypophysis still attached. We are excited about using this explant in future studies, to define causal relationships between vasopressin brain receptors, vasopressin synthesis and release, and osmotic stimuli.

One study has suggested that stimulation of renal mechanoreceptors appear to regulate the responses of vasopressin neurons in the paraventricular nucleus of the brain (Ciriello, J., 1998). We thus felt it would be interesting to determine whether up or down regulation of receptors in the kidney may influence the regulation of vasopressin receptors or vasopressin synthesis in the brain. In examining changes in vasopressin renal receptor mRNA we found that pituitary vasopressin content was indeed related to kidney V2R mRNA in control rats (r=0.60, p= 0.02), but not in alcohol-exposed rats (r=0.30, p=0.29) (fig. 8).

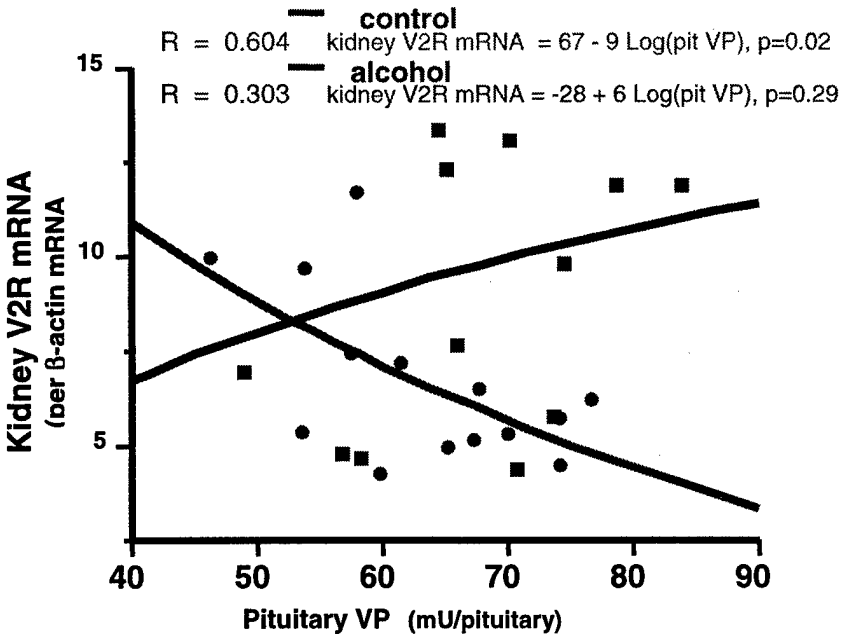

fig. 8 Relationship between pituitary VP and V2R mRNA in the kidney. Pituitary vasopressin content was related to kidney V2mRNA levels in control rats but not in alcohol-exposed rats.

It is interesting that pituitary vasopressin content appeared to be related to V2R regulation. Again, these correlations do not define a causal role V2 receptors may play in vasopressin release from pituitary stores. It is nonetheless interesting that kidney receptors may act as distant sensors to stimulate vasopressin release and decrease pituitary stores.

Measurement of kidney vasopressin receptor mRNA:

Because our results from the whole animal experiments clearly indicated that changes in renal vasopressin receptors in the different phases of alcohol exposure was likely, we first focused our attention on assessing vasopressin V2 receptor mRNA levels in the inner medullary collecting duct. Because of the highly sensitive and reproducible measurements obtained with the real time PCR technology, we were able to detect the effects of acute alcohol, chronic alcohol, and alcohol withdrawal on renal V2 receptor synthesis that would not have been as easily detected by any other method.

The results of renal V2 mRNA quantitation reported last year have been verified with additional sample analyses. V2 receptor synthesis as indicated by the V2 to β-actin mRNA ratio was significantly less in rats acutely exposed to alcohol compared to controls. A down regulation of renal V2 receptors is consistent with the increased water diuresis observed in the acute alcohol group. In contrast, renal V2 receptor mRNA was greater in the chronic alcohol exposure group compared to controls, which is consistent with an up regulation of V2 receptors causing the impaired ability to excrete a water load with chronic alcohol exposure. Lastly, during the withdrawal phase, V2 receptor mRNA returned toward normal as did water load excretion ability. Thus, these results indicate that the up regulation of renal V2 receptor mRNA seen with chronic alcohol exposure can be reversed upon withdrawal, similar to recently reported recovery of vasopressin mRNA in the brain seen with alcohol withdrawal (Silva et al, 2002).

In this third year of study we studied V2 receptor expression further by examining whether the up-regulation of V2 receptors was specific to the inner medulla of the kidney or whether there was non-specific up-regulation occurring throughout the kidney, or with other receptor subtypes. Thus, we compared (fig. 9) the levels of V1 and V2 receptor mRNA in the cortex, outer medulla, and inner medulla zones of kidneys from control (n=16) and chronic alcohol-exposed rats (n=17) using real-time PCR. Chronic alcohol-exposed caused an up-regulation of V2 receptor gene expression in the inner medulla (8.7 ± 0.9 vs 6.8 ± 0.8 relative expression units in chronic alcohol-exposed vs control, respectively, $p<0.05$), and not in the cortex or outer medulla. For both control and chronic alcohol-exposed groups, the level of V2 mRNA was consistently highest in the inner medulla and lowest in the cortex. V1 mRNA gene expression in the control and chronic alcohol-exposed groups were similar, and there were no differences between kidney zones. Results suggest that chronic alcohol specifically targets V2 receptors. This specific up-regulation of renal V2 receptor mRNA in the inner medulla is responsible for impaired water excretion seen in chronic alcohol-exposed rats.

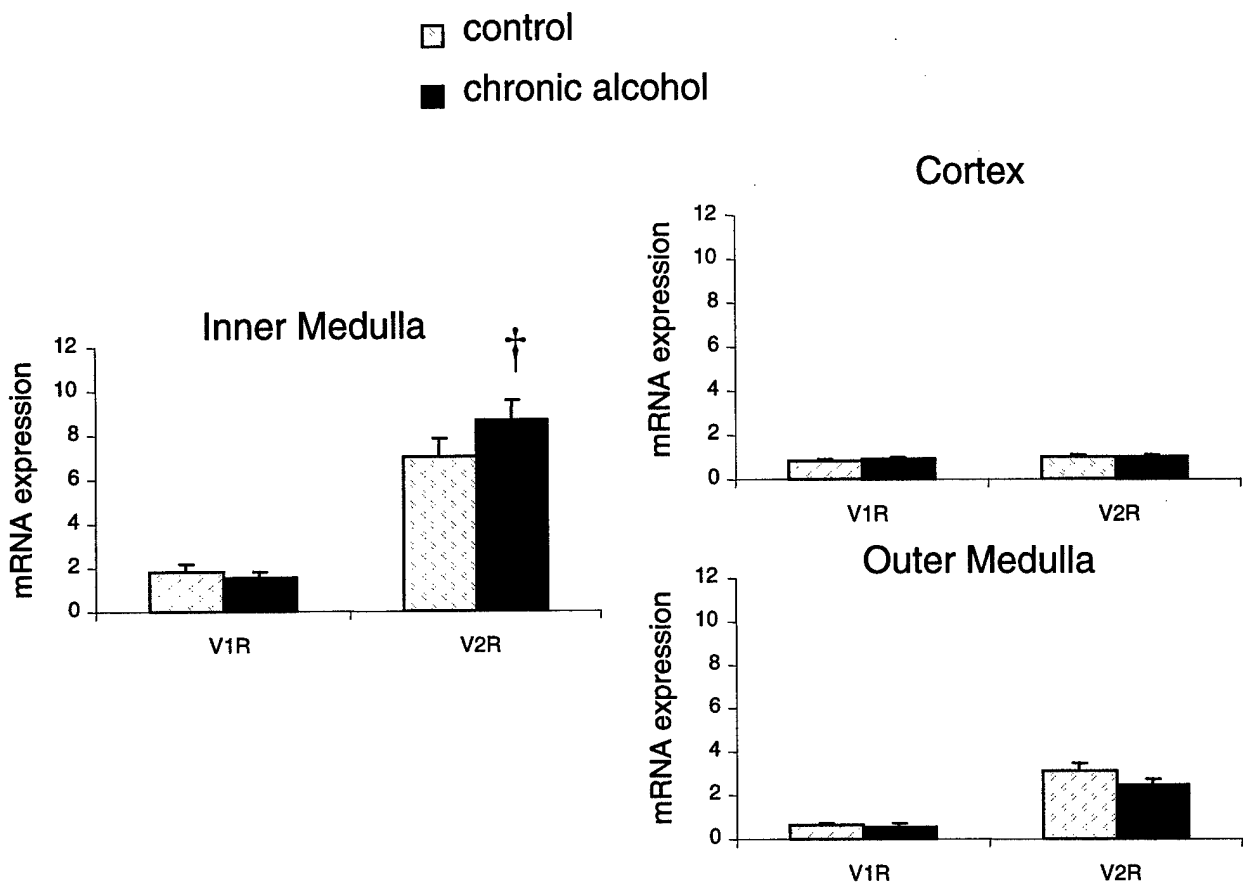

fig. 9 V1R mRNA and V2R mRNA in different regions of the kidney in control rats and rats chronically exposed to alcohol. In response to chronic alcohol exposure, V2mRNA was only up regulated in the inner medulla. This up regulation was specific to the V2 receptor as V1RmRNA was unaffected. Values represent means ± sem. + = significant difference between control and alcohol-exposed rats, $p<0.05$.

Assessment of kidney vasopressin receptor numbers and binding affinity:

While we initially intended on assessing vasopressin receptor numbers and binding affinity with traditional receptor binding methods, we have replaced the need to do so with the molecular methodology of the qPCR assays we developed. However, we needed to demonstrate that the mRNA assessments could be interpreted to directly translate into receptor protein synthesis and thus receptor numbers. In this third year we have completed binding studies assessing receptor density in acute alcohol exposure. Vasopressin V2 receptor numbers and binding affinity are in accordance with the findings of the vasopressin V2 receptor mRNA measurements. V2 receptor binding in renal inner medullary collecting duct cells obtained from rats acutely exposed to alcohol is less than that of control rats, similar to the finding of reduced V2 mRNA with acute alcohol exposure.

Assessment of kidney collecting duct cell function:

So far, our quantitative assessment of V2 receptor changes are in accordance with whole animal assessment of renal water handling abilities in all phases of alcohol exposure. Thus, it is no longer critical to verify with cell physiometry methods whether changes in receptor numbers translate into changes in renal cell function.

KEY RESEARCH ACCOMPLISHMENTS:

- Results indicate that even short-term alcohol use, equivalent to 3 days of binge drinking, can alter hydration status eighteen hours after the last alcohol drink, as water diuresis appears to persist even after blood alcohol concentrations are back to undetectable levels. This suggests that soldiers need to be adequately rehydrated after any use of alcohol to avoid fluid and electrolyte imbalances that could affect soldier performance in the field.

- Vasopressin levels are only transiently suppressed after acute alcohol exposure, and the persistent water diuresis seen 18 hours after alcohol exposure appears to be caused by a down regulation of renal V2 receptors as confirmed by V2 mRNA quantitation and receptor binding studies.

- Results suggest that long-term alcohol exposure (equivalent to about 2-3 six packs of beer a day for 8 weeks) impairs the ability of the kidneys to process water due to long term exposure to alcohol causing a compensatory up-regulation of renal receptors for vasopressin. This has implications for the effect of alcohol on the regulation of body fluid balance.

- Results indicate that the up regulation of V2 receptors during modrate chronic alcohol exposure is reversible 4 weeks after termination of alcohol exposure, indicating that impaired renal fluid handling can be reversed. This has strong implications for a recommended strategy of delaying routine field drug administration (e.g. chloroquine) for soldiers until impaired fluid handling can be reversed in order to avoid drug-induced renal toxicity that is exacerbated with alcohol.

- Results show that V1 receptors in the brain are regulated in response to changes in osmolality. This exciting finding may indicate a putative osmoreceptor role for V1 receptors in the brain or perhaps a role linking vasopressin synthesis and osmotic status.

- Results indicate that chronic alcohol causes the relationship between brain vasopressin synthesis, circulating vasopressin levels, V1 receptors, and plasma osmolality, to be disturbed. This indicates the loss of appropriate linking of the blood levels of this important water regulating hormone with the message the brain receives to synthesize the hormone in response to altered hydration status. This may have serious consequences in a soldier's ability to adjust to normal physiological stimuli such as dehydration.

- Results suggest that regulation of vasopressin secretion is altered during the withdrawal phase of alcohol exposure with the same physiological stimulus (change in blood saltiness) causing a hypersensitive release of vasopressin into the blood. This likely explains the water retention often reported during alcohol withdrawal and may cause increased likelihood of developing a dangerous state of hyponatremia and brain seizures.

REPORTABLE OUTCOMES

- Publications/Presentations
  - Published abstract and presentation at Experimental Biology 2001:
    CFT Uyehara, CA Burghardt, GM Hashiro, and DA Person. After effects of acute alcohol exposure on renal water handling and responsiveness to vasopressin.
    FASEB J. 15(4):A134 (Abstract 154.1), 2001 and
    J. Investigative Medicine 49(2):249A (Abstract 328), 2001.

  - Published abstract and presentation at Experimental Biology 2002:
    CFT Uyehara, CA Burghardt, DPY Cheng, GM, Hashiro, AK Sato, and JR Claybaugh. Chronic alcohol exposure causes impaired water excretion and decreased renal efficacy of a V2 antagonist. *FASEB J.* 16(5):A837-A838, 2002.

  - Published abstracts and presentations at Experimental Biology 2003:
    Uyehara CFT, J Wu, CA Burghardt, and AJ Marean. Alcohol withdrawal reverses increased renal vasopressin sensitivity in rats chronically exposed to alcohol. *FASEB J.* 17(5): A929 (Abstract 588.15), 2003.

    Uyehara CFT, J Wu, JR Claybaugh, AK Sato, and GM Hashiro. Alcohol exposure disrupts the relationship between brain vasopressin V1 receptors and vasopressin synthesis. *FASEB J.* 17(5): A931-A932 (Abstract 588.26), 2003.

    Wu J, GM Hashiro, and CFT Uyehara. Difference in fluid handling following acute and chronic alcohol exposure in rats is due to altered regulation of renal vasopressin V2 receptor. *FASEB J.* 17(5): A928-A929 (Abstract 588.12), 2003.

- Animal models for acute and chronic alcohol exposure and withdrawal from alcohol for precise administration of alcohol that provide a consistent response have been developed for assessment of renal fluid and electrolyte handling. These models also make efficient use of animals enabling reduction of numbers of animals used in research.

- Molecular assays utilizing quantitative PCR technology for measuring gene expression of vasopressin and vasopressin V1 and V2 receptors have been designed and established by our laboratory. These assays allow highly sensitive detection of subtle physiological changes in vasopressin receptor mRNA and protein synthesis. These assays allow uncovering physiological signals for regulation of hormone synthesis and action at the effector organ that could not previously be done with traditional methods.

- Postdoctoral fellowship pharmacology training of one new scientist, and biomedical research training of 2 undergraduate women, are supported by this award.

- Animal model and molecular biology methods established by this project have led to development of a fetal alcohol project and award of a septic shock grant used to support Army Graduate Medical Education residents and fellows.

CONCLUSIONS:

In the third year of this study, we have:

- verified the renal mechanisms behind the increased diuresis seen after acute alcohol exposure, and the impaired water excretion seen after chronic alcohol exposure, that were discovered in the first 2 years of this study

- determined that up regulation of renal V2 receptors in response to chronic alcohol is specific to the inner medullary zone of the kidney and specific to the V2 receptor, as up regulation was not seen with the renal V1 receptor nor in other regions of the kidney

- characterized the immediate effects of alcohol on the time course of fluid excretion and vasopressin secretion to clearly delineate the distinction between a transient suppression of circulating vasopressin during alcohol intake and the prolonged renal effect of water diuresis after vasopressin has returned to normal levels when there is no evidence of blood alcohol

- uncovered that a relationship between brain vasopressin receptors and plasma tonicity and vasopressin synthesis exists and that chronic alcohol exposure disrupts normal relationships between vasopressin synthesis, vasopressin release, and vasopressin receptor regulation

- revealed rebound effects in vasopressin regulation that appear to occur in response to withdrawal from chronic alcohol exposure as evidenced by a shift in a plasma osmolality-plasma vasopressin relationship, revealed by salt loading experiments

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